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REPORT ON AN INTERCALIBRATION EXERCISE ON A SHORT-TERM STANDARD TOXICITY TEST WITH ARTEMIA NAUPLII (ARC-TEST)

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ABSTRACT

Considering the necessity for standardization of toxicity tests and the need for simple and reliable routine tests, the Artemia Reference Center at the State University of Ghent in Belgium has developed a short term standard toxicity test with Artemia nauplii.

This simple and inexpensive bioassay which can be used as a screening test to determine the toxicity of chemicals to aquatic organisms, has been discussed in detail during a special workshop devoted to toxicity tests with brine shrimp, at the occasion of the "International Symposium on the brine shrimp Artemia salina" (Corpus Christi, Texas, USA ; August 1979).

Besides a few minor modifications, the test methodology was acceptable to the specialists present, who recommended that an intercalibration exercise be organized at the international level, in order to determine the reliability and the reproducibility of the test procedure.

Such an exercise has recently been organized in North America under the joint supervision of the Artemia Reference Center in Ghent, Belgium and the Toxicology Section of the Freshwater Institute in Winnipeg, Canada.

An analogous exercise was conducted in Europe by the Artemia Reference Center under the sponsorship of the Commission of the European Economic Communities.

The results of the latter ring test in which 59 laboratories from 11 countries participated are presented and commented upon.

The revised version of the standardized ARC-test, resulting from the intercalibration exercise, is included.

INTRODUCTION

Although scientific literature reveals the existence of a broad range of toxicity tests to determine the effect of chemicals or waste products on aquatic organisms it appears, in practice, that very few of these bio-assays have been standardized and that there is an urgent need for simple, reliable, inexpensive, routine tests.

The *Artemia* Reference Center (ARC) at the laboratory for Biological Research in Aquatic Pollution and Laboratory for Mariculture at the State University of Ghent in Belgium has endeavoured to develop a new "first screening" test with larvae of the brine shrimp *Artemia*. The major advantage of this bioassay is that it can be started anywhere and anywhen because of the worldwide commercial availability of *Artemia* cysts.

The nauplii which are used as test-organisms can indeed be hatched from the cysts, within 24 hours, after hydration in seawater.

This advantage completely eliminates the necessity of maintenance of live brood stock, but also makes that the *Artemia* test is extremely cheap and can be standardized to a high degree since it starts from "standard" inert dry cysts.

Departing from the existing literature on the toxic effects of some chemicals on brine shrimp, and critically reviewing various bioassays proposed so far with *Artemia* larvae, all parameters of importance were determined for a routine short-term toxicity test with an acceptable reproducibility. The factors considered in this regard were of physico-chemical, biological, technological as well as economical nature (Vanhaecke et al., 1980).

During this detailed study an experimental test protocol was worked out based on the determination of the LC50-24 hours of mixed instar II-III nauplii populations hatched out of the cysts of a standard *Artemia* strain (Vanhaecke et al., 1981). The ARC-test was discussed in detail during a special workshop on toxicity tests with brine shrimp at the occasion of the "International Symposium on the brine shrimp *Artemia salina*", Corpus Christi, Texas, USA, August 1979 (Persoone and D'Agostino, 1980).

At the end of this Convention a recommendation was formulated that the reliability and reproducibility of the ARC-test, which had received the

approval of the specialists present, should be analysed in an intercalibration exercise in order to determine the degree of standardization achieved.

Two such exercises have been organized by the Artemia Reference Center in North America and in Europe respectively. The former ring-test was set up in collaboration with the Toxicity Section of the Freshwater Institute in Winnipeg, Canada; the latter has been carried out under contract with the Commission of the European Economic Communities.

IMPLEMENTATION OF THE EUROPEAN INTERCALIBRATION EXERCISE

Approximately one hundred positive replies, originating from institutes, laboratories and firms in 13 European countries, were received to the call for participation issued by the ARC at the end of 1980.

All the interested parties were provided with the documents (instructions, experimental protocol, reply forms) and materials (artificial seawater salts, reference Artemia cysts, reference chemical[1] and unknown chemical[2]).

Each participant was requested first to carry out a preliminary test on both chemicals to determine the critical range of toxicity and then to run the definitive test 3 times, at different moments, to determine both the level of intra- and inter-laboratory variability[3]. Participants were asked to send their results, accompanied by eventual comments and criticisms to the ARC, within 6 months of time.

59 laboratories turned in their data ; the replies came from the following countries : Belgium 7, Denmark 2, France 18, Germany 8, Great Britain 6, Ireland 2, Italy 5, Netherlands 7, Norway 1, Spain 1, Sweden 2.

In order to respect the anonimity of the participants in relation to the data forwarded, each of these 59 laboratories was assigned a code number.

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[1] Sodium laurylsulphate

[2] Potassium dichromate

[3] intra-laboratory variability = based on the individual results of each participant (= repeatability)

inter-laboratory variability = based on the results obtained by different operators in different laboratories (=reproducibility)

In an attempt to find out to what extend the experimental protocol had been rigourously respected by each participant and to determine more precisely eventual weaknesses of the methodology proposed, an extended questionnaire was finally mailed to all labs that had turned in their results, and the replies considered in detail.

PROCESSING OF DATA

At the aid of Cohran's test the (few) laboratories were detected for which the intra-laboratory variation was significantly higher (at the 0.05 level) than the remainder of variations ; those results were not included in the further processing.

In a second step, Dixon's test was applied to all data to find out which of the average LC50's provided by each lab for both chemicals were significantly different from the main body of data. These "outlying" LC50 were also discarded from the total set of results for further consideration.

An analysis of variance (Model II) was then carried out on the remaining data to determine :

- the mean LC50 for the two chemicals
- the intra-laboratory standard deviation and coefficient of variation
- the inter-laboratory standard deviation and coefficient of variation

In order to obtain a frequency distribution, the data from all laboratories were classified in decreasing rank order in class sizes of 2 mg/l for Sodium laurylsulphate and 4 mg/l for Potassium dichromate respectively. Finally the median and mode were determined.

RESULTS and DISCUSSION

The results of the statistical processing of the LC50's are summarized for both chemicals in Tables 1 and 2 respectively. Two laboratories had to be excluded due to a substantial deviation from the experimental protocol with regard to the test temperature. The frequency distribution curves are given in Figures 1 and 2.

34 % of the laboratories provided data on the dissolved oxygen concentration at the end of the experiment. All of them mentioned that oxygen concentration was higher than 2 mg/l in the lowest concentration

Table 1. Statistical treatment of the data - Sodium laurylsulphate

Mean LC50-24hr	22.52 mg/l
Intra-laboratory - standard deviation	3.27 mg/l
Intra-laboratory - coefficient of variation	14.52 %
Inter-laboratory - standard deviation	5.59 mg/l
Inter-laboratory - coefficient of variation	24.82 %
Median	21.0 mg/l
Mode	17.0 mg/l
Mean f_{LC50}	1.186
Number of measurements	143
Number of laboratories excluded because of deviation from the experimental protocol	2
Number of laboratories excluded because of insufficient repeatability	4
Number of laboratories excluded because of insufficient reproducibility	0

Table 2. Statistical treatment of the data - Potassium dichromate

Mean LC50-24hr	38.87 mg/l
Intra-laboratory - standard deviation	6.65 mg/l
Intra-laboratory - coefficient of variation	14.54 %
Inter-laboratory - standard deviation	13.56 mg/l
Inter-laboratory - coefficient of variation	34.89 %
Median	36.5 mg/l
Mode	34.0 mg/l
Mean f_{LC50}	1.244
Number of measurements	146
Number of laboratories excluded because of deviation from the experimental protocol	2
Number of laboratories excluded because of insufficient repeatability	5
Number of laboratories excluded because of insufficient reproducibility	0

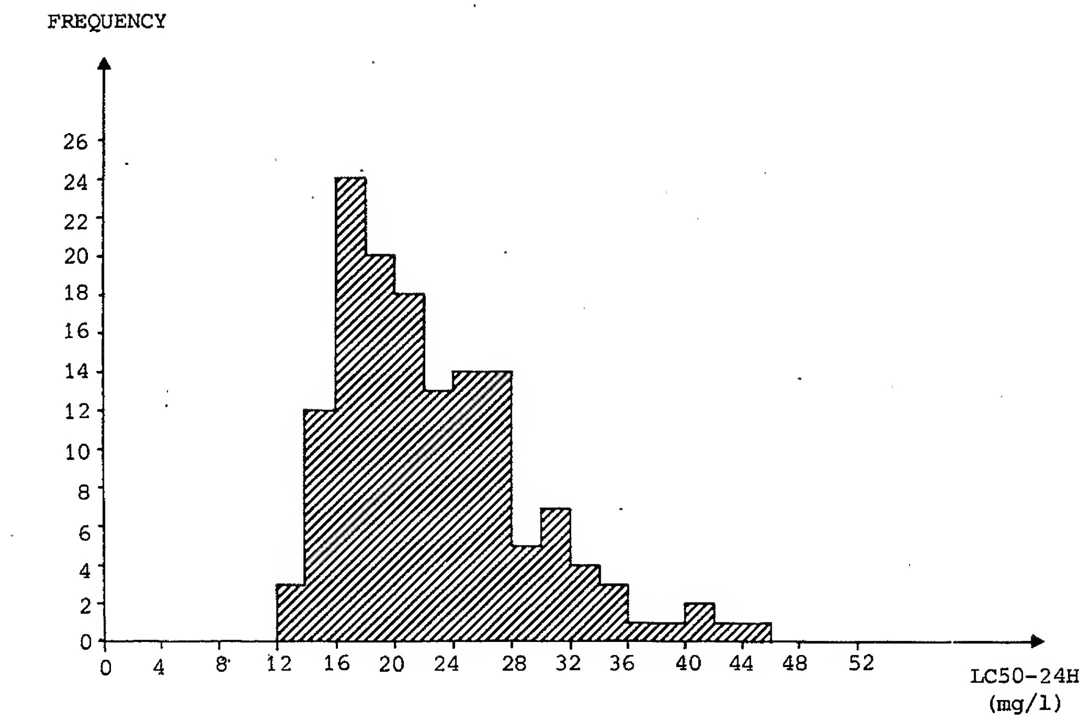

Fig. 1. Frequency distribution for the LC50 values obtained with Sodium laurylsulphate

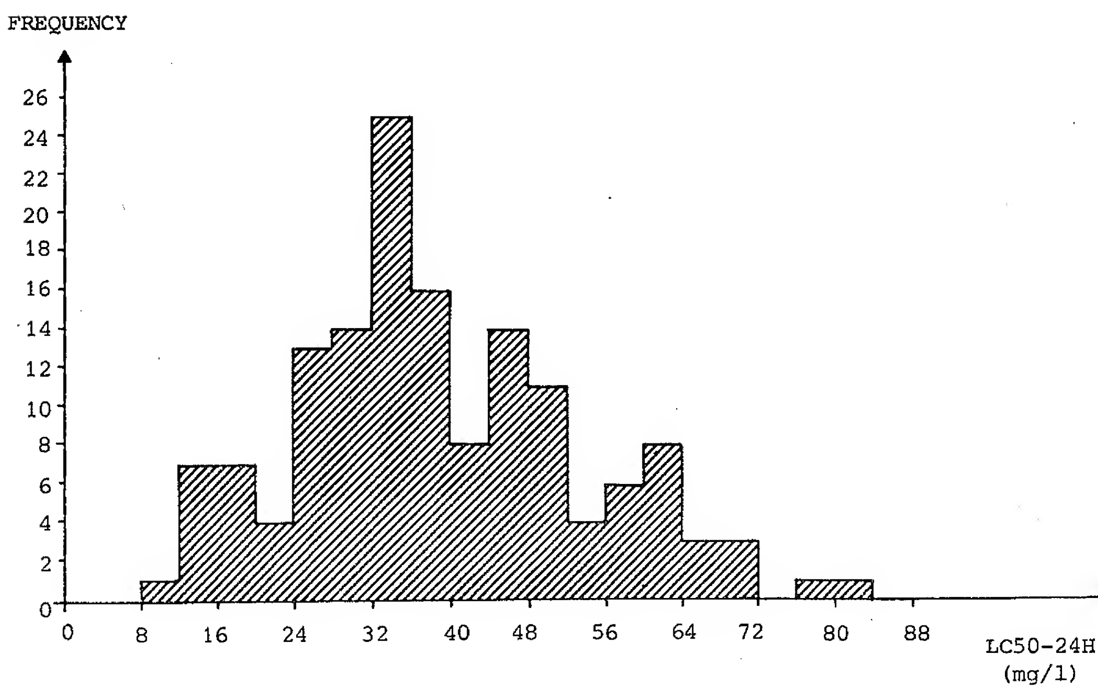

Fig. 2. Frequency distribution for the LC50 values obtained with Potassium dichromate

showing 100 % mortality of the larvae. The mean oxygen concentration reported exceeded 90 % saturation.

The mean percentage mortality in the controls was 2.7; in most cases, no mortality occurred in the controls.

The overall mean LC50-24 hour value (22.52 mg/l) for the reference chemical (Sodium laurylsulphate) is situated outside the 13.3-19.9 mg/l range proposed in the experimental protocol.

From the frequency distribution in Figure 1 it is obvious that the relatively high value of the overall mean LC50 for the reference chemical is principally due to the small number of data at the right end of the distribution curve, whereas the mode clearly fits into the range proposed.

The percentage of data situated outside the interval proposed for Sodium laurylsulphate (59 %) is comparable to the percentage recorded for the interlaboratory ring test on Daphnia (Cabridenc, 1979a) : 56 % of the latter results also fell outside the range 0.9 - 1.5 mg/l proposed by the ISO draft standard for the reference chemical.

For both Sodium laurylsulphate and Potassium dichromate the intra-laboratory coëfficient of variation is 14.5 %. From the comparison of the data for intra-laboratory variance of the intercalibration bio-assays carried out with Daphnia and Brachydanio (Cabridenc, 1979 a,b) and the value which we obtained for Artemia , it is clear that the repeatability of the Artemia test equals that of the internationally adopted Daphnia test (intra-laboratory variation for $K_2Cr_2O_7$: 14 %). The Brachydanio test did markedly better than both Daphnia and Artemia tests for the intra-laboratory variation (9.4 % for the LC50-24 hr and 7.6 for the LC50-48 hr).

The inter-laboratory coefficient of variation for $K_2Cr_2O_7$ was 34.9 %. This value is situated between those found for the Daphnia ring test (39 %) and the Brachydanio-test (22.6 - 24.7 %). However, the inter-laboratory variance for Sodium laurylsulphate (24,8 %), which is the reference chemical for the Artemia test, is much lower than the value for the reference chemical ($K_2Cr_2O_7$) with Daphnia and equals the value obtained with Brachydanio.

With regard to $K_2Cr_2O_7$ the sensibility of this short-term Artemia test (LC50-24 hour 38.9 mg/l) seems to be intermediate to that of the Daphnia test (1.42 mg/l) and the zebra-fish test (301.7 mg/l).

Although the variance of the results is quite satisfactory an analysis of the sources of variation seemed necessary to detect possible faultive manipulations made by some operators while performing the test, or weaknesses in the experimental procedure proposed.

In the first place it should be admitted that the organization of the intercalibration exercise was not perfect especially with regard to the quantity of materials sent to the different laboratories.

A substantial part of the overall variance, however, is most probably due to the fact that a lot of participants were not familiar at all with this type of bioassay. The answers to the post-exercise questionnaire indeed revealed that for 67 % of the laboratories involved this intercalibration exercise was their first contact with Artemia as a test-species for toxicological studies.

One of the most probable sources of deviation from the LC50 range proposed for the reference chemical is the fact that several labs have probably carried out the test with a mixture of instar I-II larvae, instead of instar II-III larvae as requested. Since instar I larvae are less sensitive to toxicants than instar II and instar III larvae (Sorgeloos *et al.*, 1978 ; Vanhaecke *et al.*, 1980) the mean LC50 is shifted to the right.

This error, which is easily corrected, is mainly due to the lack of expertise with this particular test-species as quoted above.

Answers to the questionnaire also revealed that some laboratories did not carry out the toxicity tests in complete conformity to the test procedure especially with regard to :

- the incubation temperature of the cysts
- the test temperature
- the renewal of the stock solution and the type of dilution water used.

Next to the reliability and sensitivity of a "first screening" eco-toxicological test its simplicity and practicality are of paramount importance (Persoone, 1979).

The answers to the questionnaire illustrated that the labor involved in carrying out the *Artemia* test is not very time consuming and almost 70 % of the participants reported that the handling and counting of the larvae takes few skill.

Furthermore the majority of participants did not seem to have major problems in carrying out the test.

In the final report submitted to the EEC (Persoone and Vanhaecke, 1981) extensive attention, comments and even statistical processing have been devoted to the answers to the post-exercise questionnaire. It is, however, outside of the scope of this brief synthesis to go into detail on this aspect of the ring-test.

The criticial analysis of the comments forwarded has, however, been very helpful in working out some minor adjustments to the original experimental protocol.

CONCLUSIONS and RECOMMENDATIONS

Considering the importance of this part of the exercise, the complete version of the "Conclusions and Recommendations" formulated in the final report to the Commission of the European Economic Communities is quoted hereunder :

The major conclusion which can be drawn from this specific international intercalibration exercise is that the short-term Artemia bioassay proposed constitutes a reliable and acceptable standardized test which will undoubtedly be welcome among the very limited number of standardized aquatic ecotoxicological tests available to date.

The fact that both the inter- and intra-laboratory variability are satisfactory in comparison to the Daphnia and Brachydanio tests is the more remarkable since contrary to the latter tests, the Artemia bioassay was entirely new to two thirds of the participating laboratories ; they were thus confronted with a totally unknown test-animal and experimental protocol.

On the basis of these facts the organizers are very confident that the variability of the test will even decrease substantially when the experimentators will have gained more experience in applying this particular bioassay.

The intercalibration exercise has been most helpful in defining some weak points of the experimental protocol ; this has led to the drafting of a revised version with minor adjustments and improvements. The new version

of the experimental protocol is added in annex and should from now on been given preference to that which served for the intercalibration exercise.

Although the Artemia short term bioassay has not been developed to replace any other aquatic test it is interesting to know its specific properties and advantages as well as it weaknesses :

1) the sensitivity of this specific test is intermediate to that of the short-term Daphnia and Brachydanio bioassays
2) its repeatability and reproducibility are at least equal to that of the Daphnia test
3) the cost is very low in comparison to any other short term bioassay with aquatic animals
4) there is no maintenance of live stock because of the availability of cysts
5) the test is applicable year round and worldwide with but a modest equipment
6) a very high degree of standardization and intercomparison of results is possible because of the worldwide availability of the same test-material.

As a result we think that this short-term bioassay is acceptable as a very suitable first screening bioassay for the marine environment and as a routine test for a first gross toxicity ranking of chemicals.

On the basis of the results of this ring-test and considering the international interest for this new bioassay, the Artemia Reference Center (ARC) will propose this aquatic ecotoxicological test - which will be called the ARC-test - for acceptance as a standard routine short term aquatic ecotoxicological test, to the International Standardization Organization, the OECD, and the Commission of the European Economic Communities.

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RESUME : RESULTATS D'UN EXERCICE D'INTERCALIBRATION SUR UN TEST STANDARD DE TOXICITE A COURT TERME, UTILISANT LES NAUPLII D'ARTEMIA

En prenant en compte, à la fois, la nécessité de standardisation d'une méthode d'essais et le besoin de tests de routine simples et reproductibles, l'Artemia Reference Center, au laboratoire de recherche biologique en pollution aquatique de l'Université de Gand (Belgique), a mis au point un test court utilisant les nauplii d'Artemia (A.R.C. test).

Ce test très simple et très bon marché, qui peut être utilisé en routine pour déterminer la toxicité des produits sur les organismes aquatiques, a été discuté en détail durant une session spéciale consacré aux tests avec Artemia lors du Symposium International sur Artemia (Corpus Christi, Texas, USA, Août 1979).

Moyennant quelques petites modifications, la méthodologie a été acceptée par les spécialistes présents qui ont demandé un exercice d'intercalibration au niveau international pour déterminer l'applicabilité et la reproductibilité de la méthode.

Un tel exercice a été organisé en Amérique du Nord sous le contrôle de l'A.R.C. de Gand et de la Section Toxicologie du Freshwater Institute de Winnipeg, au Canada.

Un exercice analogue a été exécuté en Europe par l'A.R.C. avec l'aide de la C.E.E. Les résultats de ces deux exercices seront présentés et discutés.

STANDARDIZED SHORT TERM TOXICITY TEST WITH ARTEMIA NAUPLII (ARC-TEST)

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EXPERIMENTAL PROTOCOL

1. SCOPE AND FIELD OF APPLICATION

This standard method developed at the Artemia Reference Center at the State University of Ghent in Belgium aims at determining the acute toxicity to nauplii of the brine shrimp Artemia of :

a. chemical substances

b. industrial and domestic effluents considered for dumping, or dumped into the marine environment.

2. PRINCIPLE

Determination of the concentration which kills 50% of the Artemia nauplii within 24 hours under the conditions described in the present standard. This concentration is known as the LC50-24 hr.

3. LABORATORY

The preparation of the test, the storage of the dilution water, and all stages of the test procedure described below must take place in an atmosphere free from dust and toxic vapors.

4. MATERIALS

4.1. The test organism

A homogenous population of instar II-III nauplii[1] hatched out from cysts of a well-defined Artemia strain[2] must be used to carry out the test.

4.2. Dilution water

A standard artificial seawater of 35 ± 1 °/oo is used for the hatching as well as for the test. Whenever possible, the artificial salt mixture of "Instant Ocean®" dissolved in distilled water shall be utilized.

After aeration and stabilization for 24 hours the dilution water should have a pH of 8.0 ± 0.5 and the oxygen content should be at least 90 % saturation.

If necessary the pH should be adjusted with concentrated hydrochloric acid or sodium hydroxide.

Prior to use, the water should preferably be filtered through a 1 µm filter under vacuum and aerated ; the seawater should not be stored for more than two weeks. Storage at low temperature is recommended.

4.3. Laboratory equipment

- constant temperature cabinet : 25 ± 1°C ;
- glass petri dishes (60 mm x 12 mm) with appropriate covers ;
- Pasteur pipettes with smoothed openings ;
- cylindrical (graduated cylinders) or preferably cylindroconical hatching tubes (diameter ± 35 mm) with a content of at least 100 ml ;

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(1) *Strict application of the hatching and harvesting schedule outlined in 5.1. should give a homogenous instar II-III population ; a rapid check of the larvae under a microscope may, however, be helpful in case of doubt (see Fig. 1).*

(2) *The Artemia Reference Center at the State University of Ghent, in Belgium, has accepted to act as a distribution center of "Reference Artemia Cysts" for fundamental research and for toxicity studies (Sorgeloos, Availability of Reference Artemia Cysts, Marine Ecology - Progress Series, 1980, 3, 363-364 and Aquaculture, 1981, 23, 381-382). It is advised to check cysts of unknown origin with Reference Artemia Cysts to determine if both the time schedules mentioned in this ARC-test, for hatching and molting, and the sensivity of the larvae to the reference chemical are comparable.*

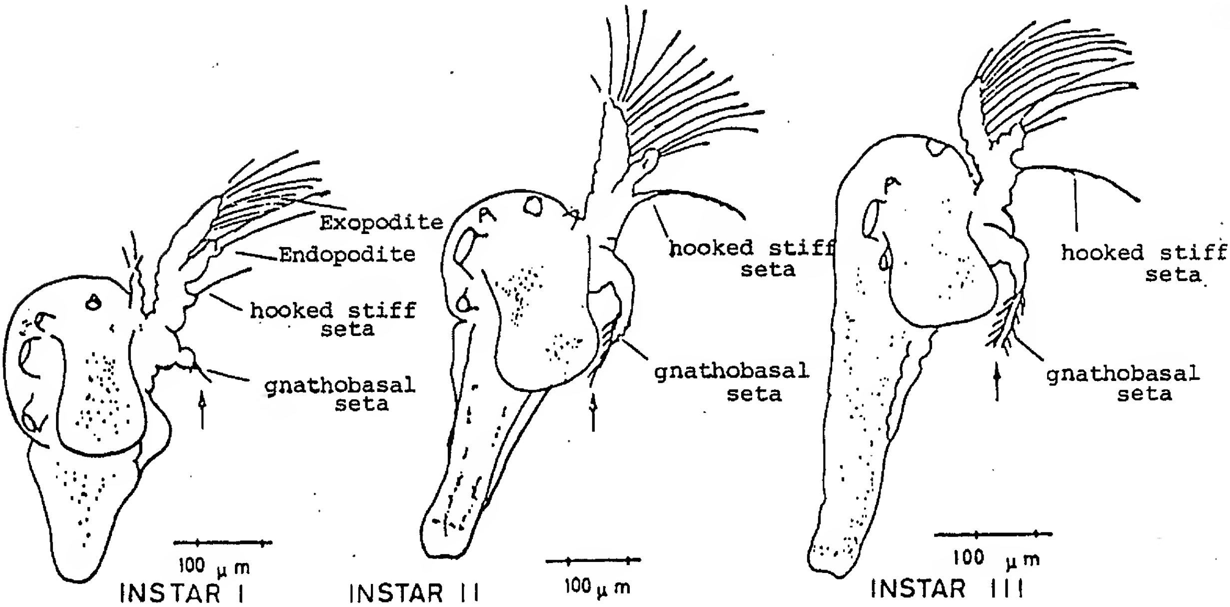

Fig. 1. Morphological characteristics of first, second and third instar nauplii of Artemia (after Hentschel, 1968)

The instar I nauplius, coloured brownisch-orange due to the presence of yolk, is pear-shaped. The gnathobasal seta is short and hairless. The hooked stiff seta and the other setae on the median and proximal segment of the 3rd antenna are also hairless.

The instar II larva is longer and paler than the instar I. Its gnathobasal seta is longer and hairy. The hooked stiff seta and the other setae of the 3rd antenna are hairy.

The instar III nauplius has a hairy gnathobasal seta which is forked terminally.

- dissolved oxygen meter ;
- binocular dissection microscope ;
- small airpump (aquarium pump) ;
- bulb or light tube ;
- usual laboratory materials.

4.4. Reference toxicant

The selected reference chemical is Sodium laurylsulphate (grade 98-102%). This compound is commonly used in surface tension research.

5. PROCEDURE

5.1. Hatching and preparation of the nauplii

For each test approximately 100 mg of cysts are incubated in 100 ml seawater in a cylindroconical tube or graduated cylinder, at a temperature of 25 $\pm$ 1°C and with lateral illumination by a bulb or light tube (intensity of at least 500 lux).

All the cysts and the hatching nauplii should be kept in continuous suspension by gentle aeration provided by a small air tube extending to the bottom of the hatching device.

After 18 up to 24 hours the aeration is stopped and the moving nauplii which concentrate at the bottom of the tube are sucked out by pipetting and transferred into an Erlenmeyer flask containing 200 ml of seawater.

This suspension should be gently aerated and kept for exactly 24 hours at a temperature of 25 $\pm$ 1°C and at an illumination of 500-1000 lux. During that time all nauplii will molt to the instar II and some of them as far as the instar III stage (see Fig. 1).

At the end of this period an aliquot of the nauplii is poured into a petri dish for subsequent manual distribution to the test petri dishes.

5.2. The toxicity test : general

The test is carried out in small glass petri dishes.

10 nauplii are transferred with a Pasteur Pipette into each dish. The volume of seawater carried over with the nauplii should not exceed 0.05 ml.

The dishes are then filled with 10 ml of the respective concentrations of the toxicant (already acclimated at 25°C), closed, and incubated in darkness at a temperature of 25 ± 1°C.

After 24 hours the number of dead larvae in each petri dish is counted under a dissection microscope. The nauplii are considered dead if no movement of the appendages is observed within 10 seconds.

Immediately after counting, the oxygen concentration is measured in the petri dish with the lowest concentration of toxicant that induced a 100% mortality. If necessary the contents of the 3 duplicate petri dishes are carefully poured together to obtain a volume large enough for the measurement.

5.3. Preliminary test

This test is performed to determine the "critical range".

A series of geometrically spaced concentrations or dilutions of the toxicant are prepared with artificial seawater.
Example for chemical substances :
    10 000, 1 000, 100, 10, 0.1, 0.01 mg/l
Example for effluents :
    100, 10, 1, 0.1, 0.01 %

The preliminary test is carried out with only one petri dish per concentration. An additional dish with 10 nauplii in 10 ml artificial seawater is included as control.

5.4. Definitive test

This test aims at the determination of the LC50-24 hr, on the basis of the critical range concentrations obtained in the preliminary test.

Concentrations and dilutions are chosen from a logarithmic scale (Douderoff et al. 1951). In principle five concentrations should be sufficient. For a satisfactory LC50, however, at least two data must be situated in the 5-95 % mortality range. If this is not the case, the test should be repeated with additional intermediate concentrations from the dilution scale.

For each concentration, including the control, three replicates should be set up.

5.5. Checking of the sensitivity of the *Artemia* nauplii and of the conformity with the experimental procedure

The LC50-24 hr. of the reference chemical Sodium laurylsulphate must be determined each time in parallel with the definitive test in order to verify the stability of the sensitivity of the experimental procedure.

The following concentrations of Sodium laurylsulphate should be tested in three replicates : 10, 13.5, 18, 24 and 32 mg/l. To prepare the 100 mg/l Sodium laurylsulphate stock solution,it is recommended to dissolve the chemical at 25°C and to use a magnetic stirrer, since the product does not dissolve quickly. The stock solution should not be stored for more than 48 hours.

6. CALCULATION AND VALIDITY OF THE RESULTS

The LC 50-24 hr. can be calculated by graphical interpolation.

The percentages of mortality between 5 and 95 % are calculated from the average number of dead nauplii per concentration, and plotted on log-probit paper. A straight line is drawn at sight through the points. The intersection of this line with the 50 % mortality horizontal line determines the LC50-24 hr.

An alternative more precise procedure is to use the method of Litchfield and Wilcoxon (1949) with which the 95 % confidence limits can be calculated.

The test can be considered valid if the following conditions are fulfilled :

- the percentage mortality in the control does not exceed 10 % ;
- the LC50-24 hr. of Sodium laurylsulphate is situated between 13.3 and 19.9 mg/l ;
- the dissolved oxygen concentration at the end of the experiment is higher than 2 mg/l in the lowest concentration with 100 % mortality of the larvae.

7. REPORTING OF THE RESULTS

The following facts shall always be reported :

- the origin of the *Artemia* strain and, if possible, the batch number of the commercial brand used ;
- the calculated LC50-24 hr., if possible with the 95 % confidence limits ;
- the critical (0-100 % mortality) range ;

- the data confirming the validity of the results ;
  a) LC50-24hr. of Sodium laurylsulphate
  b) the percentage mortality in the controls
  c) the oxygen content at the end of the test in the lowest concentration of toxicant with 100 % mortality of the larvae ;
- the method of calculation used for the determination of the LC50-24hr. ;
- any deviation from the standard procedure and any problem encountered during the test.

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